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# Narrow Spectrum Kinase Inhibitors Demonstrate Promise for the Treatment of Dry Eye Disease and Other Ocular Inflammatory Disorders

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**PURPOSE.** The purpose of this study is to determine the potential of narrow spectrum kinase inhibitors (NSKIs) to treat inflammatory eye disorders.

**METHODS.** Human conjunctival epithelial (HCE) cells were retrieved from subjects via impression cytology. Real-time quantitative PCR (qPCR) was performed on HCE cells to determine gene expression of NSKI kinase targets and proinflammatory cytokines in dry eye disease (DED) patients versus healthy controls. qPCR also assessed *p38α* expression in hyperosmolar-treated Chang conjunctival epithelial cells. Interaction of NSKI TOP1362 with the kinases was evaluated in ATP-dependent Z-LYTE and competition binding assays. Anti-inflammatory activity was assessed in human peripheral blood mononuclear cells and primary macrophages. In an endotoxin-induced uveitis (EIU) study, lipopolysaccharide (LPS) was administered intravitreally to Lewis rats. TOP1362, dexamethasone, or vehicle was administered topically, and inflammatory cytokine levels were measured 6 hours after LPS injection.

**RESULTS.** HCE cells from DED patients showed significantly increased expression of *p38α*, spleen tyrosine kinase (*Syk*), *Src*, lymphocyte-specific protein tyrosine kinase (*Lck*), interleukin one beta (*IL-1β*), interleukin eight (*IL-8*), monocyte chemotactic protein-1 (*MCP-1*), and matrix metalloproteinase-9 (*MMP-9*). TOP1362 strongly inhibited the kinase targets *p38α*, *Syk*, *Src*, and *Lck*, blocked the rise in *p38α* expression in hyperosmolar Chang cells, and potentially reduced inflammatory cytokine release in cellular models of innate and adaptive immunities. In the EIU model, TOP1362 dose-dependently attenuated the LPS-induced rise in inflammatory cell infiltration and ocular cytokine levels with efficacy comparable to that of dexamethasone.

**CONCLUSIONS.** TOP1362 is a potent inhibitor of kinases upregulated in DED and markedly attenuates proinflammatory cytokine release in vitro and in vivo, highlighting the therapeutic potential of NSKIs for treating ocular inflammation, such as that observed in DED.

**Keywords:** kinase inhibitor, dry eye disease, endotoxin-induced uveitis, inflammation

Dry eye disease (DED) is a chronic, multifactorial inflammatory disorder of the lacrimal functional unit characterized by pain, visual disturbances, tear film instability, and, in severe cases, blindness.<sup>1,2</sup> This disorder is associated with aging, contact lens wear, refractive surgery, and immune diseases, and it affects 15% to 30% of those over 50, depending on ethnicity.<sup>3,4</sup> Current therapies for treating DED are unsatisfactory. Until recently, the only approved therapy for DED was Restasis (Allergan, Irvine, CA, USA), a product whose active ingredient is the immunosuppressant cyclosporin A (CsA).<sup>5,6</sup> This treatment has limited efficacy, tolerability issues, and a slow onset of action and is licensed for treating signs of the disease only. Corticosteroids are commonly prescribed and are effective on both signs and symptoms of DED. However, these drugs are restricted to short-term use as a consequence of the serious ocular adverse effects they produce.<sup>7</sup> Recently, a new therapy, Xiidra (Shire US, Lexington, MA, USA), containing the

active ingredient lifitegrast,<sup>8</sup> has been approved for treating both the signs and symptoms of DED, but this agent also has limited efficacy and commonly occurring toleration issues.<sup>9</sup> Consequently, an unmet medical need still exists<sup>10</sup> for an innovative, fast-acting, effective, safe, and well-tolerated immunomodulatory therapy to address both the signs and symptoms of DED.

Several studies indicate that both tear film hyperosmolarity and ocular surface inflammation<sup>11,12</sup> play crucial roles in the initiation and progression of DED.<sup>13,14</sup> The evidence for an inflammatory response in the disease is provided by the presence of inflammation-related markers, including interleukin one beta (*IL-1β*), interleukin six (*IL-6*), interleukin eight (*IL-8*), interferon gamma (*IFN-γ*), matrix metalloproteinase-9 (*MMP-9*), tumor necrosis factor alpha (*TNF-α*), and monocyte chemotactic protein-1 (*MCP-1*), in tear fluids and conjunctival epithelia from patients with DED.<sup>15–25</sup> Kinases are key



TABLE 1. Clinical Data of Subjects Who Participated in Study

Sex	Age, y	OSDI	NITBUT, s	Schirmer, mm	Eye Sampled	DED (D) or Control (C)	Treatment
Female	31	31.8	5	4	Right	D	Preservative-free eyedrops
Female	22	22.7	5	8	Right	D	Eyedrops with preservative
Female	44	20.8	7	0	Right	D	Contact lens rewetting drops
Female	53	20.8	4	0	Right	D	None
Male	43	16.7	7	4	Right	D	Eyedrops with preservative
Female	42	14.6	6	2	Right	D	None
Female	27	15.0	4	5	Right	D	Eyedrops with preservative
Male	50	27.1	6	8	Right	D	Preservative-free eyedrops
Female	86	13.9	5	8	Right	D	Preservative-free eyedrops
Female	23	4.2	14	27	Right	C	None
Female	24	6.3	14	10	Right	C	None
Male	20	0.0	12	13	Right	C	None
Female	32	4.5	12	35	Right	C	None
Male	57	0.0	11	20	Right	C	None
Female	57	6.8	11	13	Right	C	None
Female	42	8.3	10	25	Left and right	C	None
Male	36	0.0	11	16	Right	C	None
Female	31	4.2	38	30	Right	C	None
Male	29	6.3	12	10	Right	C	None

mediators of cytokine production<sup>26</sup> and ocular surface stress.<sup>27</sup> As such, various inhibitors of these enzymes elicit anti-inflammatory effects in preclinical models of DED.<sup>28,29</sup> The efforts in developing kinase inhibitors to treat ocular inflammation have culminated with the topical ophthalmic Janus kinase (JAK) inhibitor tofacitinib<sup>30–32</sup> and the dual JAK-Syk inhibitor<sup>33</sup> prodrug R348 being advanced into the clinic as potential therapies for DED.

Narrow spectrum kinase inhibitors (NSKIs) are a novel class of pharmacologic agents that simultaneously target key kinases involved in both innate and adaptive immune cell signaling, namely, mitogen activated protein kinase (MAPK) p38 $\alpha$ , spleen tyrosine kinase (Syk), and Src family kinases (SFK), including Src and lymphocyte-specific protein tyrosine kinase (Lck). Recent studies have highlighted the potential of NSKIs in alleviating inflammatory conditions such as ulcerative colitis (UC),<sup>34</sup> chronic obstructive pulmonary disease, (COPD),<sup>35</sup> and arthritis.<sup>36</sup> Little is known, however, about the expression of NSKI targets in DED, with the exception of MAPK p38 $\alpha$ , which is upregulated by hyperosmolarity and desiccating stress in preclinical models.<sup>37–41</sup> Although not directly investigated hitherto, Lck and Syk are likely to be involved in the known role of T cells in DED pathogenesis,<sup>12,42</sup> a hypothesis supported by the fact that currently approved therapies for DED, Restasis and Xiidra, target T cells. Therefore, NSKIs offer a novel approach to target simultaneously both the early stages (hyperosmolarity and desiccating stress of the ocular surface) and the latter stages (dysregulated T-cell response) of DED. Here, we reveal, for the first time, increased expression of NSKI targets p38 $\alpha$ , *Syk*, and *SFK* in conjunctival impression cytology (CIC) cells from DED patients, which coincides with raised levels of inflammatory markers. Accordingly, we demonstrate that TOP1362—which combines p38 $\alpha$ , Syk, and SFK inhibitory activities within a single molecular framework—exerts potent anti-inflammatory effects in both in vitro and in vivo models of inflammatory eye disease, giving rise to the premise that NSKIs could be used to treat disorders characterized by chronic inflammation, such as DED.

## MATERIALS AND METHODS

### Subjects

The study was performed in line with research ethics permission received from GCU Life Sciences Ethics Committee (April 2014, LS1333). Informed consent was obtained from each subject. Classification of the ocular surface status of subjects was determined from the outcome of three measurements, namely ocular surface disease index (OSDI<sup>43,44</sup>), noninvasive tear break-up time (NITBUT<sup>45</sup>), and the Schirmer 1 test.<sup>46</sup> Control subjects provided OSDI scores of <10, NITBUT of  $\geq 10$  seconds, and Schirmer strip wetting length of  $\geq 10$  mm. Subjects providing OSDI scores, NITBUT, and Schirmer 1 test results outside of these ranges were classed as DED (Table 1). CIC (bulbar conjunctiva) was performed on 10 healthy controls (mean age,  $35 \pm 13$  years) and 9 DED subjects (mean age,  $44 \pm 19$  years).

### Cell Culture

The “Chang” human conjunctival cell line (Wong-Kilbourne derivative of Chang conjunctiva, clone 1-5c-4; ATCC, Manassas, VA, USA; certified cell line [CCL]-20) was cultured in standard conditions (5% CO<sub>2</sub>, 95% humidified atmosphere, at 37°C) in complete media: Dulbecco’s modified Eagle GlutaMAX medium (DMEM) enriched with 10% (v/v) heat-treated fetal bovine serum (FBS), penicillin (50 U/mL), and streptomycin (50 U/mL; all Thermo Scientific, Gillingham, United Kingdom). Cells were maintained weekly by passaging (subculturing) with 0.25% v/v trypsin-EDTA at a 1:3 ratio to maintain cell confluency.

For hyperosmolarity assays, cells were seeded at a density of  $1 \times 10^5$  cells per well in 12-well culture plates and allowed to grow to 50% to 60% confluency. Cells were then treated for 6 hours with hyperosmolar solutions of NaCl (100 and 400 mM) or sucrose (200 and 400 mM) to determine the most suitable hyperosmolar conditions. Quantitative PCR (qPCR) assays were carried out, and results were analyzed using the  $\Delta\Delta C_T$  method,  $2^{(\Delta\Delta C_T)} = \frac{(C_T \text{ target gene} - C_T \text{ GAPDH})}{(C_T \text{ target gene} - C_T \text{ GAPDH})}$ , where glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was the chosen

TABLE 2. Human Primers Used in qPCR Target Gene Analysis

Primer	Catalog No.	UniGene No.	RefSeq Accession No.	Band Size, bp	Reference Position
<i>p38<math>\alpha</math> MAPK</i>	330001 PPH00750B	Hs.485233	NM_001315.2	90	690
<i>Syk</i>	330001 PPH01639F	Hs.371720	NM_003177.5	119	1818
<i>Src</i>	330001 PPH00103C	Hs.195659	NM_005417.4	131	1366
<i>Lck</i>	330001 PPH00185E	Hs.470627	NM_005356.3	132	941
<i>IL-1<math>\beta</math></i>	330001 PPH00171C	Hs.126256	NM_000576.2	126	574
<i>IL-8</i>	330001 PPH00568A	Hs.624	NM_000584.3	126	326
<i>MCP-1</i>	330001 PPH00192F	Hs.303649	NM_002982.3	122	106
<i>MMP-9</i>	330001 PPH00152E	Hs.297413	NM_004994.2	63	2107
<i>GAPDH</i>	330001 PPH00150F	Hs.592355	NM_002046.5	130	842

housekeeping gene. Target genes were standardized to *GAPDH*. Assays were carried out in triplicate.

### Eyeprim Sample Collection

Human conjunctival epithelial cells (HCEs) were retrieved from healthy controls ( $n = 10$ ) and subjects with DED ( $n = 9$ ), using the Eyeprim device (Opia Technologies, Paris, France). Membranes were transferred to sterile eppendorf tubes with RLT (RNA lysis buffer; Qiagen, Manchester, United Kingdom) containing  $\beta$ -mercaptoethanol and stored at  $-80^{\circ}\text{C}$  until processing for RNA extraction. Two presses per membrane were taken each at temporal and inferior conjunctiva of subjects.

### Total RNA Extraction

Total RNA was extracted from Chang cells and human CIC samples with the QIAshredder and RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Total RNA, extracted from Chang cells ( $6 \times 10^6$  cells/mL) and human CIC samples, was quantified and assessed for purity with the NanoDrop spectrophotometer (Thermo Scientific).

### Reverse Transcription of Total RNA

Complementary DNA (cDNA) was synthesized from total RNA and extracted with the RT<sup>2</sup> First Strand Kit (Qiagen), following the manufacturer's instructions. Synthesized cDNA was stored at  $-20^{\circ}\text{C}$  until required for qPCR analysis.

### Real-Time PCR

Details of the primers used (Qiagen) are provided in Table 2.

The qPCR was carried out as follows for one reaction: 12.5  $\mu\text{L}$  RT<sup>2</sup> SYBR Green Mastermix (Qiagen), 1  $\mu\text{L}$  RT<sup>2</sup> qPCR Primer Assay (10  $\mu\text{M}$  stock; Qiagen), 10.5  $\mu\text{L}$  RNase-free water (Qiagen), and 1  $\mu\text{L}$  cDNA template (500 ng/ $\mu\text{L}$ , unless otherwise stated) for both Chang cells and human CIC samples. Reactions were performed in duplicate using 96-well plates (Thermo Scientific). RNase-free water was used as negative template controls. Using the ABI 7500ViiA qPCR machine (Applied Biosystems, Warrington, United Kingdom; or Bio-Rad, Hercules, CA, USA), the PCR reaction was incubated at  $95^{\circ}\text{C}$  for 15 minutes, followed by 39 cycles of  $95^{\circ}\text{C}$  for 15 seconds, and  $60^{\circ}\text{C}$  for 20 seconds, and then the plate was read. A dissociation curve, involving the incubation of the formed product for an additional 30 minutes at  $60^{\circ}\text{C}$ , was generated to ensure only one specific product was obtained. For these assays, *GAPDH* was chosen as the housekeeping gene to which all target genes were normalized. Results were analyzed using the  $\Delta\text{CT}$  method, as mentioned above.

### Statistical Analyses

Results are presented from three independent experiments, unless stated otherwise, and are shown as mean  $\pm$  SEM. Statistical analyses were performed using GraphPad Prism V software (GraphPad, La Jolla, CA, USA). Student's *t*-test (two-tailed) and Dunnett tests were performed.

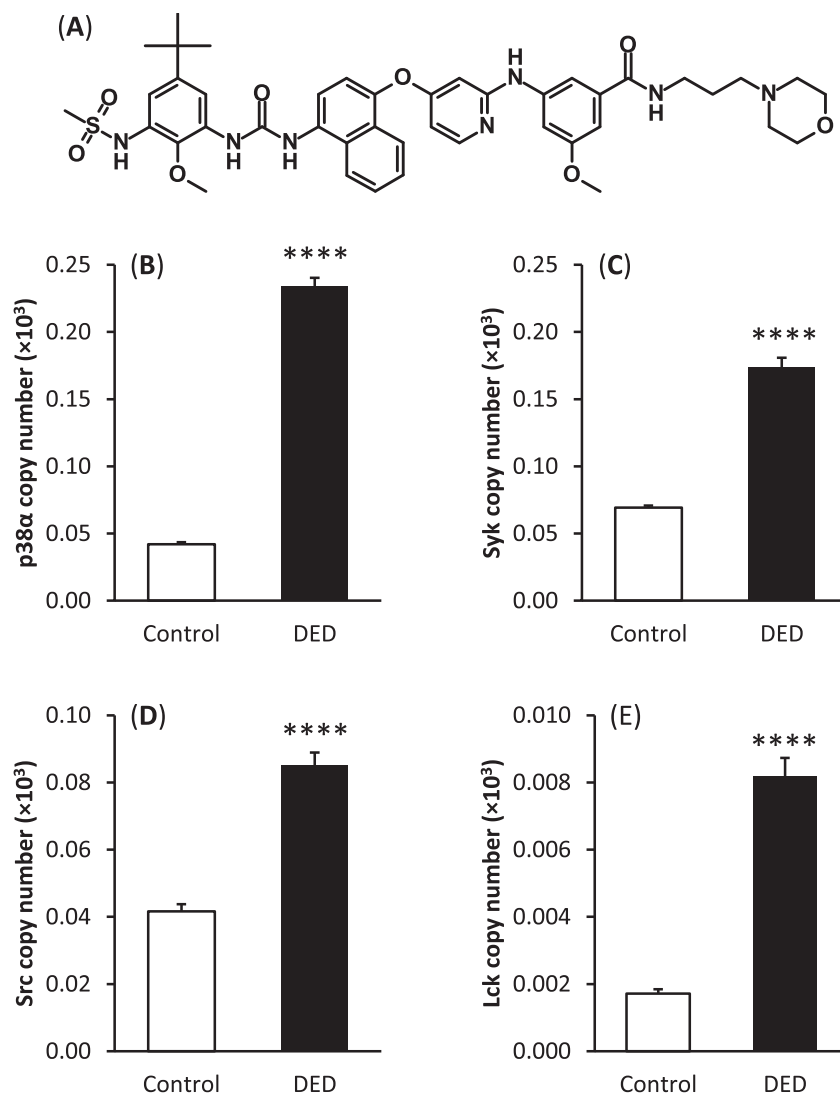
### Ligand Dissociation Kinase Assay

Kinase-tagged T7 phage strains were prepared in an *Escherichia coli* host derived from the BL21 strain. *E. coli* were grown to log-phase and infected with T7 phage and incubated with shaking at  $32^{\circ}\text{C}$  until lysis. The lysates were centrifuged and filtered to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated small molecule ligands for 30 minutes at room temperature to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer (SeaBlock; [Pierce Biotechnology, Rockford, IL, USA], 1% BSA, 0.05% Tween 20, 1 mM dithiothreitol [DTT]) to remove unbound ligand and to reduce nonspecific binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1 $\times$  binding buffer (20% SeaBlock, 0.17 $\times$  PBS, 0.05% Tween 20, 6 mM DTT). Test compounds were prepared as 111-fold stocks in 100% dimethyl sulfoxide (DMSO).  $K_d$ s were determined using an 11-point threefold compound dilution series with three DMSO control points. All compounds for  $K_d$  measurements are distributed by acoustic transfer (noncontact dispensing) in 100% DMSO. The compounds were then diluted directly into the assays such that the final concentration of DMSO was 0.9%. All reactions were performed in polypropylene 384-well plates at a final volume of 0.02 mL. The assay plates were incubated at room temperature with shaking for 1 hour, and the affinity beads were washed with buffer (1 $\times$  PBS, 0.05% Tween 20). The beads were then resuspended in elution buffer (1 $\times$  PBS, 0.05% Tween 20, 0.5  $\mu\text{M}$  nonbiotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR.

### Substrate Phosphorylation Kinase Assay

A commercial 384-well fluorescence resonance energy transfer (FRET)-based kinase assay for p38 $\alpha$ , Syk, and Src kinases was used to measure inhibitory activity of substrate phosphorylation. TOP1362 or vehicle (DMSO, 1% v/v) was incubated with the kinase of interest (p38 $\alpha$ , 50 ng/mL; Syk, 500 ng/mL; or Src, 750 ng/mL) for 2 hours. Z-lyte peptide (Invitrogen, Paisley, United Kingdom), selective for an individual kinase, was added





**FIGURE 1.** (A) Structure of TOP1362. (B) *p38α* MAPK, (C) *Syk*, (D) *Src*, and (E) *Lck* gene expression in controls ( $n = 7$ ) and DED subjects ( $n = 8$ ) via CIC sampling. Data were analyzed by the  $\Delta\Delta C_T$  method. \*\*\*\* $P < 0.0001$  comparing controls with DED.

(Ser threonine 4 peptide for p38 $\alpha$ , Tyr2 peptide for Syk and Src). ATP, 10, 15, and 200  $\mu$ M for p38 $\alpha$ , Syk, and Src, respectively, was then added. In addition, inactive MAPK-activated protein kinase (MAPKAP) 2 (180 ng/mL) was added to the p38 $\alpha$  reaction mixture. After 1-hour incubation, development reagent was added followed by an additional hour of incubation. The reaction was terminated and read using a fluorescence microplate reader.

### Preparation of Human Peripheral Blood Mononuclear Cells

Blood was diluted (1:1) with PBS-containing EDTA (2 mM) before overlaying on Lymphoprep. The sample was centrifuged (1200g, 20 minutes), and the resultant "buffy coat," containing the peripheral blood mononuclear cells (PBMCs), was collected. After further centrifugation (780g, 10 minutes) and washing ( $\times 2$ ) in PBS containing EDTA (2 mM), the final cell pellet was resuspended in 10 mL PBS/EDTA (2 mM)/BSA (0.5%). Cells were counted, and the final concentration was adjusted to  $10^6$  cells/mL by resuspension in RPMI 1640 culture media containing heat inactivated FBS (10% v/v), penicillin/

streptomycin, and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (25 mM).

### Cellular Assays

**Lipopolysaccharide Stimulation of PBMCs.** PBMCs (200  $\mu$ L) were incubated (2 hours, 37°C, 5% CO<sub>2</sub>) with test compound (0.1 to 1000 ng/mL) or vehicle (DMSO, 0.5% v/v). Lipopolysaccharide (LPS; 1 ng/mL) was added, and, after a 24-hour incubation (37°C, 5% CO<sub>2</sub>), plates were centrifuged (780g, 2 minutes). Supernatants were collected for analysis of IL-8 and TNF- $\alpha$  levels.

**Anti-CD3/Anti-CD28 Stimulation of PBMCs.** PBMCs (200  $\mu$ L) were added to anti-CD3 (clone OKT3, 0.6  $\mu$ g/mL) and anti-CD28 (clone CD28.2, 12  $\mu$ g/mL) coated 96-well plates. Compound (0.1 to 1000 ng/mL) or vehicle (DMSO, 0.5% v/v) was added. After a 48-hour incubation (37°C, 5% CO<sub>2</sub>), plates were centrifuged (780g, 2 minutes), before the collection of supernatants for analysis of IFN- $\gamma$  levels.

**IFN- $\gamma$  Release From Anti-CD3/IL-2-Stimulated PBMC Cells.** PBMCs were added to a 96-well plate, and compound (0.1 to 1000 ng/mL) was added 2 hours prior to stimulation with a mixture of monoclonal antibody to CD3 (1  $\mu$ g/mL;

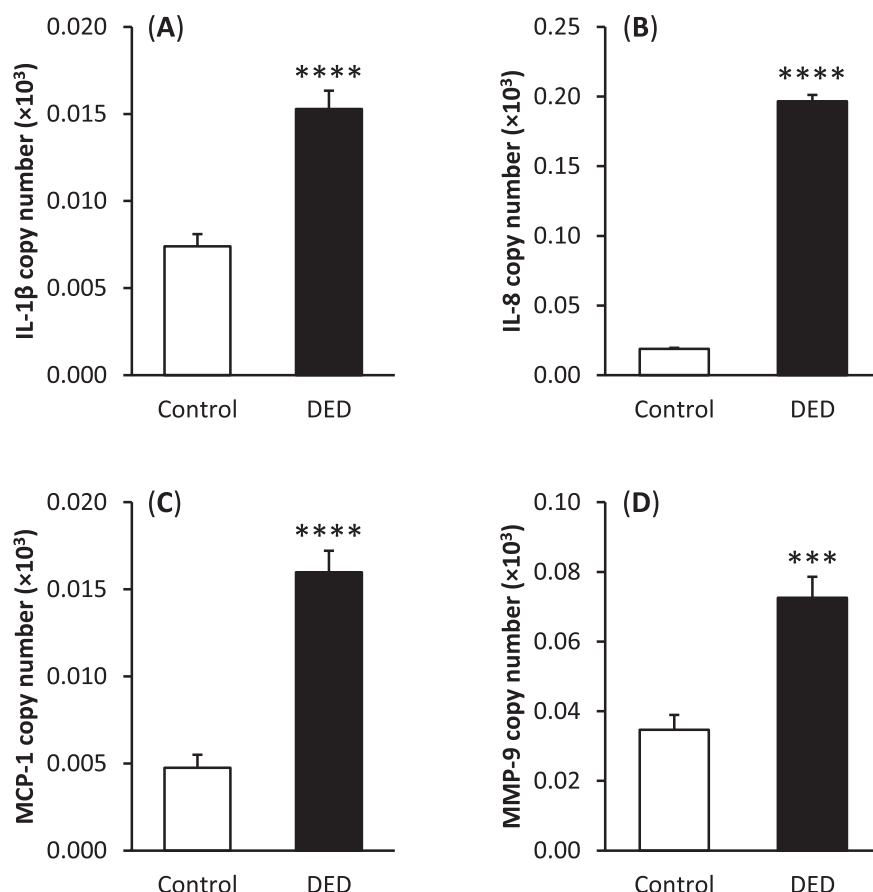


FIGURE 2. (A) *IL-1 $\beta$* , (B) *IL-8*, (C) *MCP-1*, and (D) *MMP-9* gene expression in controls and DED subjects. Data were analyzed by the  $\Delta$ CT method, where target gene expression was standardized to a GAPDH housekeeping gene. \*\*\* $P$  < 0.001 and \*\*\*\* $P$  < 0.0001 comparing controls with DED.

eBioscience, Hatfield, United Kingdom) and human recombinant IL-2 (10 ng/mL; Peprotech, Rocky Hill, NJ, USA). After 48-hour incubation under control tissue culture conditions, supernatants were collected, and IFN- $\gamma$  release was determined by Sandwich ELISA (Duo-set; R&D Systems, Abingdon, United Kingdom). IC<sub>50</sub>s were determined from the dose-response curves.

#### LPS Activation of Monocyte-Derived Macrophages.

CD14<sup>+</sup> cells were isolated from human PBMCs by positive selection using magnetic beads. Cells were resuspended in RPMI containing 10% FBS and cultured (37°C, 5% CO<sub>2</sub>) in the presence of human recombinant granulocyte-macrophage colony-stimulating factor (100 ng/mL) for 12 to 14 days. They were then harvested and resuspended ( $2 \times 10^5$  cells/mL), dispensed into 96-well plates (100  $\mu$ L/well), and allowed to equilibrate for 2 hours. Test compound (0.1 to 1000 ng/mL) or vehicle (DMSO, 0.5% v/v) was incubated with cells (2 hours) before stimulation with LPS (10 ng/mL) for 24 hours. Supernatants were collected for IL-8 and TNF- $\alpha$  analysis.

**ELISA.** All cytokines were measured using commercial ELISA kits, according to manufacturers' instructions.

**Dose Response of TOP1362 on Hyperosmolar-Stressed Chang Cells.** To assess the effect of TOP1362 treatment on *p38 $\alpha$*  gene expression, Chang cells were seeded and grown as above. These cells were exposed to a hyperosmolar solution of complete media containing NaCl (100 mM) for 6 hours, in the presence and absence of serial dilutions of TOP1362 (0.01, 0.1, and 1.0  $\mu$ g/mL). All serial dilutions were made in 0.5% DMSO, and controls (vehicle alone) contained complete media and 0.5% DMSO. Cells were

preincubated with TOP1362 (or vehicle) for 2 hours prior to hyperosmolar stress. Gene expression levels of *p38 $\alpha$*  in NSKI hyperosmolar-stressed Chang cells were determined by qPCR assays, results were analyzed using the  $\Delta$ CT method,  $2^{-(\Delta\text{CT})} = (CT_{\text{target gene}} - CT_{\text{GAPDH}})$ , and *GAPDH* was the chosen housekeeping gene to which *p38 $\alpha$*  was normalized.

#### Rat Endotoxin-Induced Uveitis

Endotoxin-induced uveitis (EIU)<sup>47</sup> was induced in male Lewis rats (6 to 8 weeks old; Charles River UK Limited, Margate, United Kingdom) through a single intravitreal administration of 100 ng/animal of LPS (*E. coli* 0111:B4, prepared in PBS; Sigma-Aldrich, Gillingham, United Kingdom) into the right vitreous humor (5- $\mu$ L dose volume) using a 32-gauge needle. Control rats were injected with PBS. Nonfasted rats were administered a solution of TOP1362, dexamethasone, or vehicle (20% hydroxypropyl- $\beta$ -cyclodextrin, 0.1% hydroxypropyl methylcellulose, 0.01% benzalkonium chloride, 0.05% EDTA, 0.7% sodium chloride in deionized water) by topical administration onto the right eye (10  $\mu$ L) at -1, 0, 1, 2, and 4 hours with respect to the LPS administration. In a separate time course experiment, TOP1362 administration took place at the different times (see Fig. 7A). Six hours after LPS dosing, animals were euthanized by overdose with pentobarbitone (via cardiac puncture). To measure cellular infiltration, 10  $\mu$ L aqueous humor was collected from the right eye of the rats immediately after euthanasia by puncture of the anterior chamber using a 32-gauge needle under a surgical microscope. The aqueous humor collected was diluted in 20  $\mu$ L PBS, and

**TABLE 3.** In Vitro Activities (nM) of NSKI TOP1362 at p38 $\alpha$ , Syk, Src, and Lck Kinases

Assay	p38 $\alpha$	Syk	Src	Lck
Ligand dissociation ( $K_d$ )	26	18	ND	3.7
Substrate phosphorylation (IC <sub>50</sub> )	137	314	32	ND

ND, not determined.

total cell counts were measured immediately using a Countess automated cell counter (Invitrogen), with data being reported as total cell numbers per milliliter. Cytokine analysis was performed on eye homogenate. Following collection of the aqueous humor, the right eye was enucleated and then dissected into front and back sections around the lens. Each section was weighed and homogenized in 500  $\mu$ L sterile PBS followed by 20 minutes centrifugation at 12,000 rpm at 4°C. The resulting supernatant was divided into three aliquots and stored at -80°C until analysis, wherein levels of IL-1 $\beta$ , IL-6, and MCP-1 were measured in duplicate using ELISA quantikine assays (R&D Systems), as per the manufacturer's instructions. All work adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

## Materials and Reagents

TOP1362, 3-((4-((4-(3-(5-(*tert*-butyl)-2-methoxy-3-(methylsulfonylamido)phenyl)ureido)naphthalen-1-yl)oxy)pyridin-2-yl)amino)-5-methoxy-*N*-(3-morpholinopropyl)benzamide (Fig. 1A), was prepared as described previously.<sup>48</sup> Z-lyte assay materials and heat-inactivated FBS were obtained from Invitrogen. Lymphoprep was obtained from Alere Limited (Stockport, United Kingdom). Anti-CD3 and anti-CD28 antibodies were supplied by eBioscience and BD Bioscience (Oxford, United Kingdom), respectively. ELISA kits were acquired from R&D Systems. All other reagents were obtained from Sigma-Aldrich.

## RESULTS

### Upregulation of p38 $\alpha$ MAPK, Syk, Src, and Lck Gene Expression in DED CIC Samples

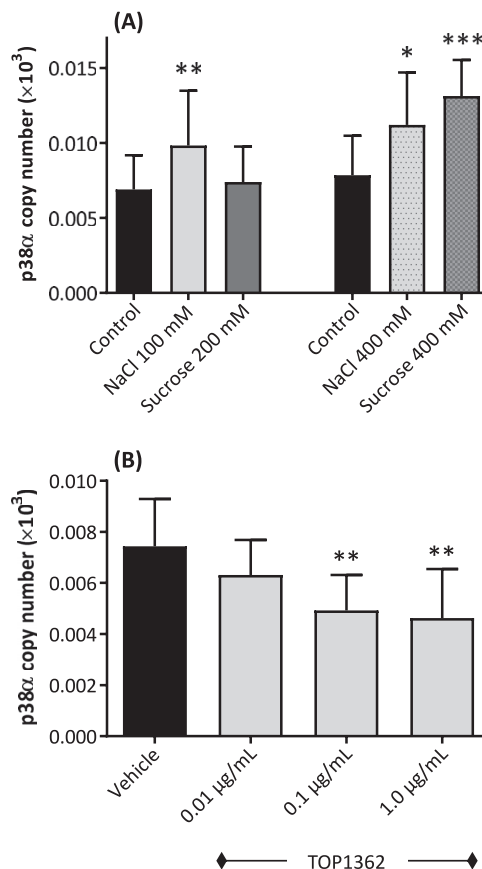
qPCR analysis was performed to quantify target gene expression differences between conjunctival cells from controls ( $n = 7$ ) and DED ( $n = 8$ ) subjects, using CIC for sample retrieval. The assays, standardized to *GAPDH* housekeeping gene, revealed significant upregulation of p38 $\alpha$  MAPK, Syk, Src, and Lck genes in subjects with DED versus control subjects (Fig. 1).

### Increased IL-1 $\beta$ , IL-8, MCP-1, and MMP-9 Gene Expression in Human CIC Samples

In subjects where there was sufficient mRNA remaining from the kinase study, qPCR analysis was performed to quantify cytokine and MMP-9 gene expression differences between controls ( $n = 3$ ) and DED ( $n = 3$ ) subjects. Consistent with reports in the literature, inflammatory markers IL-1 $\beta$ ,<sup>15,19</sup> IL-8,<sup>19</sup> MCP-1,<sup>16</sup> and MMP-9<sup>24</sup> were significantly upregulated in individuals with DED compared with control subjects (Fig. 2).

### TOP1362 Potently Inhibits p38 $\alpha$ , Syk, Src, and Lck In Vitro

TOP1362 displayed high affinity to the target kinases p38 $\alpha$ , Syk, and Lck in competition binding and ligand dissociation assays (Table 3) with dissociation constants ( $K_d$ ) of 3.7 to 26



**FIGURE 3.** (A) Expression of p38 $\alpha$  in hyperosmolar-stressed Chang cells and (B) concentration-dependent inhibition by TOP1362 of 100 mM sodium chloride-induced p38 $\alpha$  expression at 6 hours. Data were analyzed by the  $\Delta$ CT method, where target gene expression was standardized to the housekeeping gene *GAPDH*. Assays were carried out in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

nM. The potent binding observed in these assays translated into strong inhibitory activity in ATP-dependent substrate phosphorylation assays, where TOP1362 inhibited all three kinases examined (p38 $\alpha$ , Syk, and Src) with nanomolar IC<sub>50</sub>s.

### TOP1362 Concentration-Dependently Lowers p38 $\alpha$ Expression in Hyperosmolar-Stressed Chang Conjunctival Epithelial Cells

qPCR assays were carried out to assess the expression levels of the p38 $\alpha$  gene in Chang cells following hyperosmolar stress. Stress was induced using sodium chloride (100 and 400 mM) or sucrose (200 and 400 mM), for 6 hours, versus untreated control cells. After 6-hour treatment, both concentrations of sodium chloride and the higher concentration of sucrose significantly upregulated p38 $\alpha$  gene expression in Chang cells (Fig. 3A). It was observed, however, that the samples treated with the higher concentration (400 mM) of sodium chloride and sucrose had increased cell detachment and cell death. Therefore, 100 mM sodium chloride was selected for the NSKI studies.

The ability of TOP1362 to attenuate the upregulation of p38 $\alpha$  gene expression in Chang cells exposed to a hyperosmolar stress for 6 hours was evaluated. At 0.1 and 1  $\mu$ g/mL, TOP1362 significantly downregulated p38 $\alpha$  gene expression in



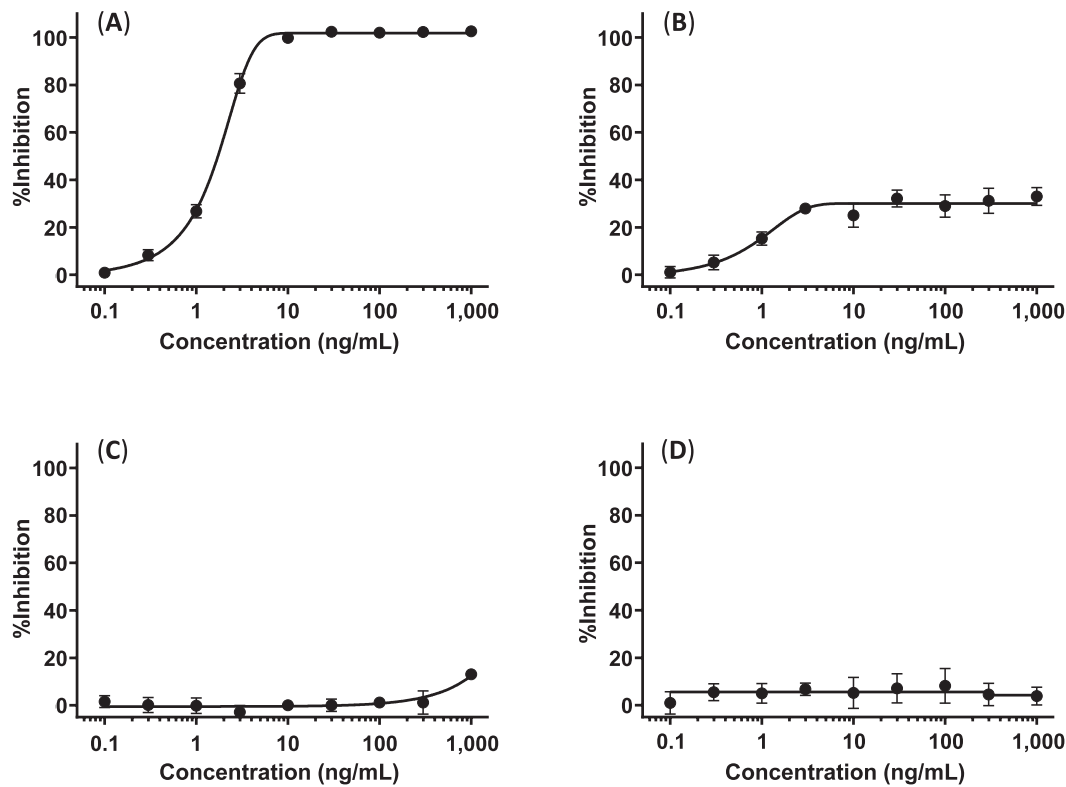


FIGURE 4. Inhibition of LPS-stimulated IL-8 release from PBMCs by (A) TOP1362, (B) budesonide, (C) tofacitinib, and (D) CsA. Associated IC<sub>50</sub> values are given in Table 4.

sodium chloride stimulated Chang cells, whereas no effect was seen at 0.01  $\mu$ g/mL (Fig. 3B).

**Inhibitory Effects of TOP1362 on Innate and Adaptive Cellular Responses**

Dry eye inflammation involves the interplay of innate and adaptive immune mechanisms within the ocular surface.<sup>12,42,49</sup> As a model of innate immunity, PBMCs were stimulated with LPS, leading to marked IL-8 release (15,700  $\pm$  1500 pg/mL). In contrast to the corticosteroid budesonide, JAK inhibitor tofacitinib and DED treatment CsA, TOP1362 produced concentration-dependent (0.1 to 1000 ng/mL), potent, and maximal (100%) inhibition of LPS-stimulated IL-8 release (Fig. 4). Furthermore, TOP1362 and budesonide both inhibited LPS-stimulated TNF- $\alpha$  release from PBMCs with

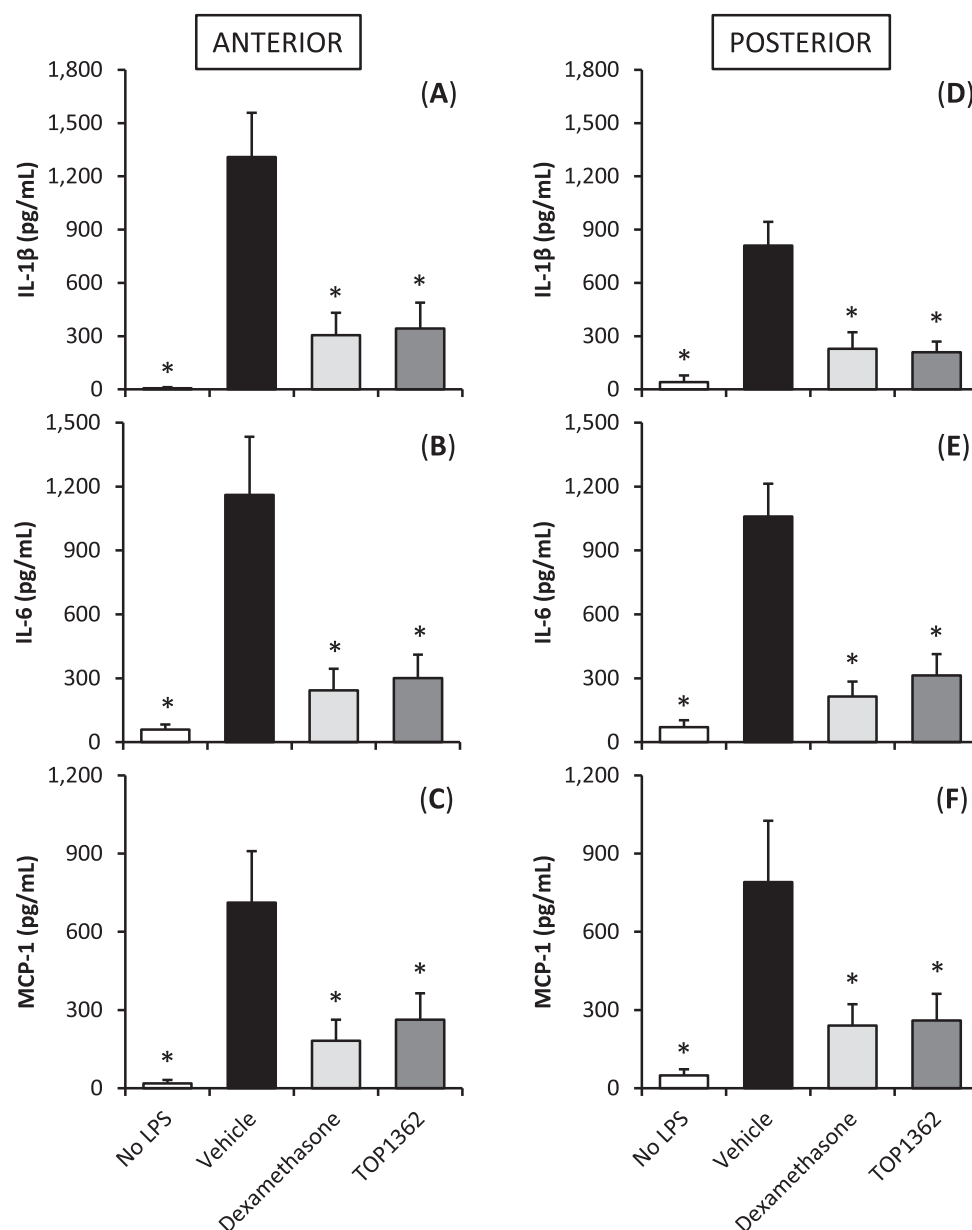
single-digit nM IC<sub>50</sub>s (Table 4), in contrast to tofacitinib and CsA, which did not inhibit release of this cytokine. A similar profile was observed in LPS-stimulated primary human macrophages, with TOP1362 achieving potent, maximal inhibition of IL-8 and TNF- $\alpha$  release, budesonide acting most effectively on TNF- $\alpha$ , and tofacitinib and CsA being largely inactive.

To model the adaptive immune response, PBMCs were stimulated with (1) anti-CD3/anti-CD28 and (2) anti-CD3/IL-2 to activate the T-cell population, resulting in release of IFN- $\gamma$  of (1) 16100  $\pm$  5900 pg/mL and (2) 2010  $\pm$  480 pg/mL. In both cases, TOP1362 achieved maximal inhibition of IFN- $\gamma$  release with low nanomolar IC<sub>50</sub>s (Table 4). The other agents evaluated also inhibited IFN- $\gamma$  release, although less potently than TOP1362, with the exception of budesonide inhibition of IFN- $\gamma$  secretion from anti-CD3/IL-2-activated PBMCs.

TABLE 4. Inhibition of Cytokine Release, Induced by Various Stimuli, From PBMCs and Primary Macrophages by TOP1362, Budesonide, Tofacitinib, and CsA

Cell Type	Stimulus	Cytokine	IC <sub>50</sub> s (nM)			
			TOP1362	Budesonide	Tofacitinib	CsA
PBMC	LPS	IL-8	1.9	>2300	>3200	>830
PBMC	LPS	TNF- $\alpha$	0.8	6.0	>3200	>830
Primary macrophage	LPS	IL-8	9.1	>580	>800	NT
Primary macrophage	LPS	TNF- $\alpha$	2.1	11	>800	NT
PBMC	$\alpha$ -CD3/CD28	IFN- $\gamma$	12	>2300	607	51
PBMC	$\alpha$ -CD3/IL-2	IFN- $\gamma$	1.6	1.2	56	9.2

NT, not tested.



**FIGURE 5.** In LPS-exposed rats, following topical ocular administration, 0.1% TOP1362 solution reduces IL-1 $\beta$  (A, D), IL-6 (B, E), and MCP-1 (C, F) levels in the anterior (A–C) and posterior (D–F) segment of the eye as effectively as an equivalent, and maximally effective, dose of dexamethasone. \* $P < 0.05$  relative to vehicle control.

### TOP1362 Is Efficacious in the Rat EIU Model

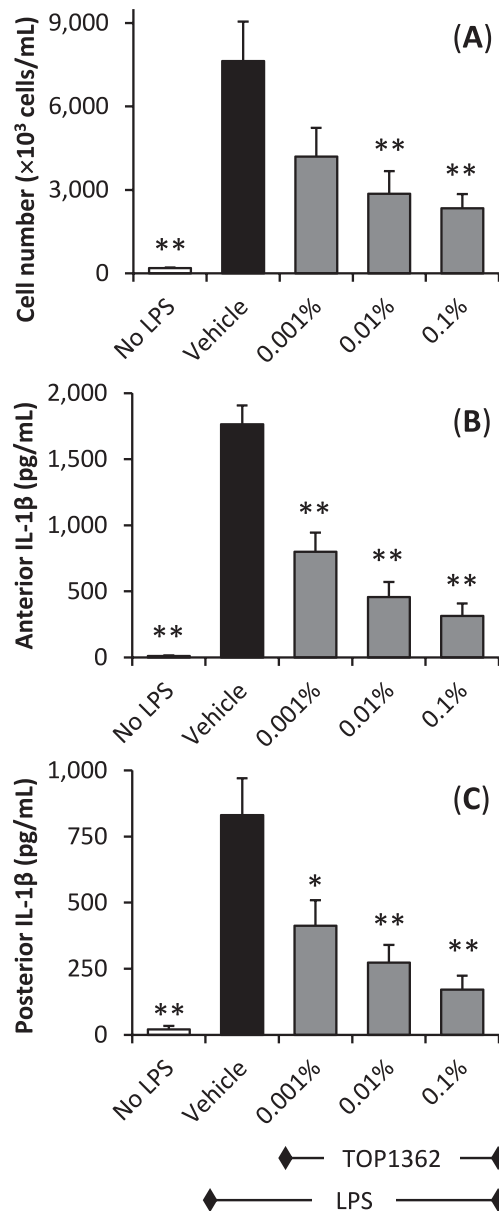
Intravitreal injection of LPS into the aqueous humor of male Lewis rats increased concentrations of the inflammatory markers IL-1 $\beta$ , IL-6, and MCP-1 in both the anterior and posterior segments of the eye (Fig. 5). Topical administration of 0.1% TOP1362 eye drops at  $-1$ ,  $0$ ,  $1$ ,  $2$ , and  $4$  hours with respect to the LPS administration attenuated the increases in these cytokines as effectively as an equivalent  $0.1\%$  dose of the corticosteroid dexamethasone (administered under the same dosing regimen).

In a separate EIU experiment, TOP1362 eye drops concentration-dependently lowered inflammatory cell infiltration into the aqueous humor (Fig. 6A). The reduction in inflammatory cell infiltrate observed correlated with decreases in IL-1 $\beta$  levels in both the anterior and posterior segments of

the eye and was dependent on the number of instillations of TOP1362 (Fig. 7).

### DISCUSSION

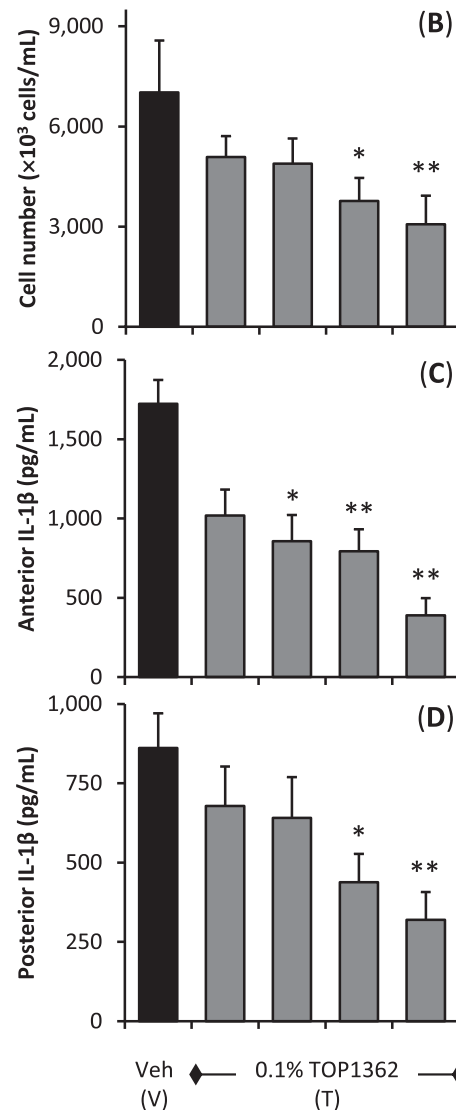
Despite recent additions to the ophthalmologist's therapeutic options (e.g., lifitegrast), the treatment of DED remains a significant unmet medical need,<sup>10</sup> and new therapies are urgently required to treat this debilitating condition characterized by chronic inflammation.<sup>50</sup> Given their potent and broad-ranging anti-inflammatory effects in a wide range of cell types with key roles in various inflammatory disorders (UC,<sup>34</sup> COPD,<sup>35</sup> and arthritis<sup>36</sup>), we hypothesized that NSKIs could address the poorly served medical need of DED. Although the initial ocular surface insult in DED can be multifactorial (low



**FIGURE 6.** Following topical ocular administration, in the rat EIU model, TOP1362 solution concentration-dependently reduced (A) the total number of cells in the aqueous humor, as well as IL-1β levels in the anterior (B) and posterior (C) segments of the eye. \**P* < 0.05 and \*\**P* < 0.01 relative to vehicle control.

tear volume, poor tear quality, contact lens use), the vicious cycle of chronic disease progression appears to be similar in most cases, with innate immune pathways becoming activated initially. Subsequently, an adaptive immune response, which becomes dysregulated ultimately, is triggered.<sup>12</sup> Intracellular kinases are involved in signaling cascades of both the innate and adaptive immune responses. To the best of our knowledge, however, no investigative studies in DED patients have been conducted to determine the expression of kinases in disease. Here, we demonstrate, for the first time, that the NSKI targets *p38α*, *Syk*, *Lck*, and *Src* are significantly upregulated in cells obtained from the ocular surface of DED patients compared with those of healthy controls. Moreover, we show that this upregulation is associated with increases in the inflammatory cytokines *IL-1β*, *IL-8*, and *MCP-1*, as well as in the inflamma-

Treatment regimen (A)				
+1h	-	-	-	T
0h	-	-	T	T
-1h	V	-	T	T
-4h	-	T	-	-



**FIGURE 7.** Anti-inflammatory efficacy of topical 0.1% TOP1362 solution in the rat EIU model employing different treatment regimens. (A) Treatment regimens used with timings expressed relative to LPS administration at T = 0 hours: V, vehicle administered; T, TOP1362 administered (single 40-μL drop); -, no dosing. (B–D) Effect of the different treatment regimens on the total number of cells in the aqueous humor (B), as well as anterior (C) and posterior (D) IL-1β levels. \**P* < 0.05 and \*\**P* < 0.01 relative to vehicle control.

tory mediator *MMP-9*. Due to limited patient samples, only *p38α* phosphorylation was investigated by immunohistochemistry and found to be upregulated in patient conjunctival cells compared with healthy volunteers (Supplementary Materials). The role of *p38α* in DED pathogenesis is further supported by its upregulation in a conjunctival cell line (Chang cells) in

response to hyperosmolar challenge, a response that can be inhibited by treatment with NSKI TOP1362.

In terms of cellular expression, p38 $\alpha$  and Src are expressed ubiquitously, whereas Syk and Lck are primarily expressed in lymphocytes. Thus, the increased expression of Syk and Lck observed in DED patients may be a reflection of a raised inflammatory infiltrate into conjunctival tissue. Although inhibitors targeting a single kinase, such as p38 $\alpha$ , have been proposed as potential therapies for DED,<sup>51</sup> these selective agents have displayed disappointing efficacy in clinical trials in other inflammatory disorders.<sup>52</sup> To obtain superior efficacy in the clinic, polypharmacology<sup>53</sup>—using a broader, multi-targeted approach—is emerging as a new drug treatment paradigm for treating diseases of complex pathophysiology, such as DED and UC.

As a consequence of its polypharmacologic mode of action, NSKI TOP1362 demonstrated wide-ranging and potent inhibition of cytokine release from several cell types induced by various different stimuli. Underscoring the potential of NSKIs as novel DED therapies, the inhibition elicited by TOP1362 was, in many cases, superior to that achieved by the topical corticosteroid budesonide. Furthermore, TOP1362 provided a much broader, and more potent, inhibition of cytokine release compared with both CsA, the active pharmaceutical ingredient in the approved DED therapy Restasis,<sup>54</sup> and tofacitinib, a JAK inhibitor with some evidence of efficacy in the clinic.<sup>30</sup>

Further evidence for the potential of TOP1362 in inflammatory eye disease was furnished by an EIU experiment in rats. The EIU model has been used<sup>47</sup> to determine the potential therapeutic efficacy of drugs that could prevent or ameliorate ocular inflammation. In this model, following topical administration, TOP1362 dose-dependently attenuated the increases in inflammatory cell infiltration and cytokines, which are shown here to be upregulated in DED patients. Interestingly, the reduction of cytokine release in the posterior segment of the eye was comparable to that elicited in the anterior segment, indicating that TOP1362 can reach the back of the eye, presumably via the trans-scleral route,<sup>55</sup> and highlighting the potential for treating posterior segment disorders. Importantly, the efficacy elicited by 0.1% TOP1362 in this experiment was almost identical to that produced by 0.1% dexamethasone over several separate EIU studies. Topical corticosteroids, such as methylprednisolone<sup>56</sup> or loteprednol etabonate,<sup>57</sup> are arguably the most effective therapies for treating DED, but they are typically administered for only a limited period of time, because they are associated with significant side effects (e.g., bacterial infection, IOP elevation, and cataract formation) after prolonged use. Interestingly, in a cellular setting, topical corticosteroid was more broad acting than either CsA or tofacitinib and was most similar, in terms of potency and breadth of activity, to TOP1362. The breadth of activity achieved by TOP1362 stems from its ability to simultaneously target multiple key signalling kinases involved in both innate and adaptive immune pathways and highlights the potential of NSKIs to treat inflammatory disorders of complex pathophysiology, such as DED. In this regard, optimization of TOP1362 gave rise to TOP1630, an NSKI that has recently provided positive results in a phase 2a clinical trial in DED (clinical study reference NCT03088605).

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### References

1. Schein OD, Tielsch JM, Muñoz B, Bandeen-Roche K, West S. Relation between signs and symptoms of dry eye in the elderly: a population-based perspective. *Ophthalmology*. 1997;104:1395–1401.
2. Begley CG, Chalmers RL, Abetz L, et al. The relationship between habitual patient-reported symptoms and clinical signs among patients with dry eye of varying severity. *Invest Ophthalmol Vis Sci*. 2003;44:4753–4761.
3. Tomlinson A, Lemp MA, Asbell PA. *Dry Eye Disease*. New York: Thieme; 2007:1–15.
4. Farrand KF, Fridman M, Stillman IO, Schaumberg DA. Prevalence of diagnosed dry eye disease in the United States among adults aged 18 years and older. *Am J Ophthalmol*. 2017;182:90–98.
5. Schultz C. Safety and efficacy of cyclosporine in the treatment of chronic dry eye. *Ophthalmol Eye Dis*. 2014;6:37–42.
6. Ames P, Galor A. Cyclosporine ophthalmic emulsions for the treatment of dry eye: a review of the clinical evidence. *Clin Invest*. 2015;5:267–285.
7. Colligris B, Crooke A, Huete-Toral F, Pintor J. An update on dry eye disease molecular treatment: advances in drug pipelines. *Expert Opin Pharmacother*. 2014;15:1371–1390.
8. Keating GM. Lifitegrast ophthalmic solution 5%: a review in dry eye disease. *Drugs*. 2017;77:201–208.
9. Donnenfeld ED, Karpecki PM, Majumdar PA, et al. Safety of lifitegrast ophthalmic solution 5.0% in patients with dry eye disease: a 1-year, multicenter, randomized, placebo-controlled study. *Cornea*. 2016;35:741–748.
10. Chao W, Belmonte C, Benitez Del Castillo JM, et al. Report of the inaugural meeting of the TFOS i(2) = initiating innovation series: targeting the unmet need for dry eye treatment. *Ocul Surf*. 2016;14:264–316.
11. Wei Y, Asbell PA. The core mechanism of dry eye disease (DED) is inflammation. *Eye Contact Lens*. 2014;40:248–256.
12. Stevenson W, Chauhan SK, Dana R. Dry eye disease: an immune-mediated ocular surface disorder. *Arch Ophthalmol*. 2012;130:90–100.
13. Enríquez-de-Salamanca A, Castellanos E, Stern ME, et al. Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. *Mol Vis*. 2010;16:862–873.
14. Baudouin C, Aragona P, Messmer EM, et al. Role of hyperosmolarity in the pathogenesis and management of dry eye disease: proceedings of the OCEAN group meeting. *Ocul Surf*. 2013;11:246–258.
15. Niu L, Zhang S, Wu J, Chen L, Wang Y. Upregulation of NLRP3 inflammasome in the tears and ocular surface of dry eye patients. *PLoS One*. 2015;10:e0126277.
16. Na KS, Mok JW, Kim JY, Rho CR, Joo CK. Correlations between tear cytokines, chemokines, and soluble receptors and clinical severity of dry eye disease. *Invest Ophthalmol Vis Sci*. 2012;53:5443–5550.
17. Zahir-Jouzani F, Atyabi F, Mojtavavi N. Interleukin-6 participation in pathology of ocular diseases. *Pathophysiology*. 2017;24:123–131.
18. Lam H, Bleiden L, de Paiva CS, Farley W, Stern ME, Pflugfelder SC. Tear cytokine profiles in dysfunctional tear syndrome. *Am J Ophthalmol*. 2009;147:198–205.
19. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of inflammatory cytokines in the tears of dry eye patients. *Cornea*. 2009;28:1023–1027.
20. Huang JF, Zhang Y, Rittenhouse KD, Pickering EH, McDowell MT. Evaluations of tear protein markers in dry eye disease:



- repeatability of measurement and correlation with disease. *Invest Ophthalmol Vis Sci.* 2012;53:4556-4564.
21. Pflugfelder SC, De Paiva CS, Moore QL, et al. Aqueous tear deficiency increases conjunctival interferon- $\gamma$  (IFN- $\gamma$ ) expression and goblet cell loss. *Invest Ophthalmol Vis Sci.* 2015;56:7545-7550.
  22. Meadows JF, Dionne K, Nichols KK. Differential profiling of T-cell cytokines as measured by protein microarray across dry eye subgroups. *Cornea.* 2016;35:329-335.
  23. Jackson DC, Zeng W, Wong CY, et al. Tear interferon-gamma as a biomarker for evaporative dry eye disease. *Invest Ophthalmol Vis Sci.* 2016;57:4824-4830.
  24. Aragona P, Aguenouz M, Rania L, et al. Matrix metalloproteinase 9 and transglutaminase 2 expression at the ocular surface in patients with different forms of dry eye disease. *Ophthalmology.* 2015;122:62-71.
  25. Caffery BE, Joyce E, Heynen ML, Ritter R, Jones LA, Senchyna M. Quantification of conjunctival TNF- $\alpha$  in aqueous-deficient dry eye. *Optom Vis Sci.* 2014;91:156-162.
  26. Li DQ, Luo L, Chen Z, Kim HS, Song XJ, Pflugfelder SC. JNK and ERK MAP kinases mediate induction of IL-1 $\beta$ , TNF- $\alpha$  and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp Eye Res.* 2006;82:588-596.
  27. Pflugfelder SC, de Paiva CS, Tong L, Luo L, Stern ME, Li D-Q. Stress-activated protein kinase signaling pathways in dry eye and ocular surface disease. *Ocul Surf.* 2005;3(4 Suppl):S154-S157.
  28. Zoukhri D, Macari E, Choi SH, Kublin CL. c-Jun NH2-terminal kinase mediates interleukin-1 $\beta$ -induced inhibition of lacrimal gland secretion. *J Neurochem.* 2006;96:126-135.
  29. Droy-Lefaix MT, Bueno L, Caron P, Belot E, Roche O. Ocular inflammation and corneal permeability alteration by benzalkonium chloride in rats: a protective effect of a myosin light chain kinase inhibitor. *Invest Ophthalmol Vis Sci.* 2013;54:2705-2710.
  30. Liew SH, Nichols KK, Klammer KJ, Li JZ, Zhang M, Foulks GN. Tofacitinib (CP-690,550), a Janus kinase inhibitor for dry eye disease: results from a phase 1/2 trial. *Ophthalmology.* 2012;119:1328-1335.
  31. Huang JF, Yafawi R, Zhang M, et al. Immunomodulatory effect of the topical ophthalmic Janus kinase inhibitor tofacitinib (CP-690,550) in patients with dry eye disease. *Ophthalmology.* 2012;119:e43-50.
  32. Stevenson W, Sadrai Z, Hua J, et al. Effects of topical Janus kinase inhibition on ocular surface inflammation and immunity. *Cornea.* 2014;33:177-183.
  33. Kheirkhah A, Di Zazzo A, Satitpitakul V, Fernandez M, Magilavy D, Dana R. A pilot randomized trial on safety and efficacy of a novel topical combined inhibitor of Janus kinase 1/3 and spleen tyrosine kinase for GVHD-associated ocular surface disease. *Cornea.* 2017;36:799-804.
  34. Biancheri P, Foster MR, Fyfe MC, et al. Effect of narrow spectrum versus selective kinase inhibitors on the intestinal proinflammatory immune response in ulcerative colitis. *Inflamm Bowel Dis.* 2016;6:1306-1315.
  35. Onions ST, Ito K, Charron CE, et al. Discovery of narrow spectrum kinase inhibitors: New therapeutic agents for the treatment of COPD and steroid-resistant asthma. *J Med Chem.* 2016;59:1727-1746.
  36. To WS, Aungier SR, Cartwright AJ, et al. Potent anti-inflammatory effects of the narrow spectrum kinase inhibitor RV1088 on rheumatoid arthritis synovial membrane cells. *Br J Pharmacol.* 2015;172:3805-3816.
  37. Luo L, Li DQ, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates MAPK signaling pathways on the ocular surface. *Invest Ophthalmol Vis Sci.* 2004;45:4293-4301.
  38. Liu H, Begley C, Chen M, et al. A link between tear instability and hyperosmolarity in dry eye. *Invest Ophthalmol Vis Sci.* 2009;50:3671-3679.
  39. Cavet ME, Harrington KL, Ward KW, Zhang JZ. Mapracorat, a novel selective glucocorticoid receptor agonist, inhibits hyperosmolar-induced cytokine release and MAPK pathways in human corneal epithelial cells. *Mol Vis.* 2010;16:1791-1800.
  40. Cavet ME, Harrington KL, Vollmer TR, Ward KW, Zhang J-Z. Anti-inflammatory and anti-oxidative effects of the green tea polyphenol epigallocatechin gallate in human corneal epithelial cells. *Mol Vis.* 2011;17:533-542.
  41. Wang C, Shi X, Chen X, et al. 17- $\beta$ -estradiol inhibits hyperosmolarity-induced proinflammatory cytokine elevation via the p38 MAPK pathway in human corneal epithelial cells. *Mol Vis.* 2012;18:1115-1122.
  42. El Annan J, Chauhan SK, Ecoiffier T, Zhang Q, Saban DR, Dana R. Characterization of effector T cells in dry eye disease. *Invest Ophthalmol Vis Sci.* 2009;50:3802-3807.
  43. Walt JG, Rowe MM, Stern KL. Evaluating the functional impact of dry eye: the Ocular Surface Disease Index. *Drug Inf J.* 1997;31:1436.
  44. Grubbs JR Jr, Tolleson-Rinehart S, Huynh K, Davis RM. A review of quality of life measures in dry eye questionnaires. *Cornea.* 2014;33:215-218.
  45. Hirji N, Patel S, Callander M. Human tear film pre-rupture phase time (TP-RPT) - a non-invasive technique for evaluating the pre-corneal tear film using a novel keratometer mire. *Ophthalmol Physiol Opt.* 1989;9:139-142.
  46. Kasetsuwan N, Satitpitakul V, Changul T, Jariyakosol S. Incidence and pattern of dry eye after cataract surgery. *PLoS One.* 2013;8:e78657.
  47. Kanai K, Ito Y, Nagai N, et al. Effects of instillation of eyedrops containing disulfiram and hydroxypropyl- $\beta$ -cyclodextrin inclusion complex on endotoxin-induced uveitis in rats. *Curr Eye Res.* 2012;37:124-131.
  48. Baker TM, Fyfe MCT, Harbottle GW, et al., inventors; Respivert Limited, Topvert Pharma Limited, assignees. Kinase inhibitors. US patent 9,481,648. November 1, 2016.
  49. Na KS, Hwang KY, Lee HS, Chung SH, Mok JW, Joo CK. Wakayama symposium: interface between innate and adaptive immunity in dry eye disease. *BMC Ophthalmol.* 2015;15(Suppl 1):159.
  50. Chiaradia PA, Zeman-Bardeci LA, Dankert S, Mendaro MO, Grzybowski A. Hot topics in dry eye disease. *Curr Pharmaceut Des.* 2017;23:608-623.
  51. Ma X, Zou J, He L, Zhang Y. Dry eye management in a Sjögren's syndrome mouse model by inhibition of p38-MAPK pathway. *Diagnost Pathol.* 2014;9:5.
  52. Goldstein DM, Kuglstat A, Lou Y, Soth MJ. Selective p38 $\alpha$  inhibitors clinically evaluated for the treatment of chronic inflammatory disorders. *J Med Chem.* 2010;53:2345-2353.
  53. Morphy R. Selective multitargeted drugs. In: Peters J-U, ed. *Polypharmacology in Drug Discovery*. Hoboken, NJ: Wiley; 2012:247-262.
  54. Utine CA, Stern M, Akpek EK. Clinical review: topical ophthalmic use of cyclosporin A. *Ocul Immunol Inflamm.* 2010;18:352-361.
  55. Doukas J, Mahesh S, Umeda N, et al. Topical administration of a multi-targeted kinase inhibitor suppresses choroidal neovascularization and retinal edema. *J Cell Physiol.* 2008;216:29-37.
  56. Marsh P, Pflugfelder SC. Topical nonpreserved methylprednisolone therapy for keratoconjunctivitis sicca in Sjögren syndrome. *Ophthalmology.* 1999;106:811-816.
  57. Sheppard JD, Comstock TL, Cavet ME. Impact of the topical ophthalmic corticosteroid loteprednol etabonate on intraocular pressure. *Adv Ther.* 2016;33:532-552.